AD_____

Award Number: DAMD17-02-1-0556

TITLE: A Novel Method to Screen for Dominant Negative ATM Mutations in Familial Breast Cancer

PRINCIPAL INVESTIGATOR: Kum Kum Khanna, Ph.D.
Georgia Chenevix-Trench, Ph.D.
Sean Grimmond, Ph.D.

CONTRACTING ORGANIZATION: Queensland Institute of Medical Research
Herston, Brisbane Qld 4029, Australia

REPORT DATE: April 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2005	Final (1 Apr 02 - 31 Mar 05)

4. TITLE AND SUBTITLE
A Novel Method to Screen for Dominant Negative ATM Mutations in Familial Breast Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0556

6. AUTHOR(S)
Kum Kum Khanna, Ph.D.
Georgia Chenevix-Trench, Ph.D.
Sean Grimmond, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Queensland Institute of Medical Research
Herston, Brisbane Qld 4029, Australia

E-Mail: leeC@qlmr.edu.au

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The aim of this proposal is to identify families carrying potentially pathogenic *ATM* mutations by assaying for ATM kinase activity in cell lines derived from individuals with multiple cases of breast cancer in their family but no pathogenic *BRCA1* or *BRCA2* mutation ('BRCAx' families). We have analysed 252 cell lines established from index cases from BRCAx families for ATM expression and activity. Overall, analysis of the data suggests that impaired activation of ATM can not be used as a screening tool to identify families that carry dominant negative mutation in ATM due to natural variation amongst LCLs. Microarrays have been performed to determine differences in gene expression between heterozygotes or homozygotes for the T7271G mutation against each other and wild type control pools. These data have suggested candidate genes that are altered in their expression in *ATM* mutation carriers. The validation of this data in carriers of different ATM mutation indicated that the heterozygous carriers of T7271G mutation display a gene expression phenotype that appears identical to carriers of protein truncating mutations in *ATM,* suggesting that the expression signature may not be specific for this particular mutation in ATM but rather can be used to assess the downstream effects of any germ-line change in the ATM gene.

14. SUBJECT TERMS
Familial breast cancer, ATM mutations, ATM activity, cDNA microarray

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The *ATM* gene is mutated in the autosomal recessive disorder, ataxia telangiectasia (A-T), which is characterised by cancer predisposition, cerebellar ataxia and immunodeficiency. One of the most controversial topic in breast cancer genetics is whether mutations in the *ATM* gene predispose women to breast cancer. Studies of A-T families appear to have an elevated frequency of breast cancer in females, particularly in obligate heterozygotes whose risk may be increased as much as 7-fold. By contrast, most studies of sporadic breast cancer have not found an increased frequency of germline *ATM* mutations compared with controls, and linkage analysis of markers close to *ATM* in multiple-case families has provided no evidence that the *ATM* gene predisposes women to breast cancer. Nevertheless, two recurrent *ATM* mutations, T7271G and IVS10-6T->G, were recently reported to be associated with breast cancer (Stankovic *et al.*, 1998; Broeks *et al.*, 2000). We analysed these two pathogenic mutation in ATM in female-breast cancer only, non-*BRCA1/2* families in the Australian based Kathleen Cunningham Foundation Consortium for Research into Familial Breast Cancer (kConFab) and observed that 3% of the families carried one of the two mutations in *ATM* analysed (Chenevix-trench et al, 2002). We have shown that both mutations act as dominant negatives in that ATM kinase activity was markedly reduced in the heterozygous carriers of these mutations. These observations suggest that a proportion of hereditary breast cancer may be due to *ATM* mutations, and that the increased breast cancer risk may be restricted to a subset of *ATM* mutations.

The aim of this proposal is to identify families carrying potentially pathogenic *ATM* mutations by assaying for ATM kinase activity in cell lines derived from individuals with multiple cases of breast cancer in their family but no pathogenic BRCA1 or BRCA2 mutation ('BRCAx' families). In addition, we aim to identify target genes dysregulated by *ATM* mutations, and ideally to develop a novel method of high throughput screening for *ATM* mutations.

Our hypotheses were that:

a) Impaired activation of ATM kinase in lymphoblastoid cell lines (LCLs) from index cases of multiple case non-BRCA1/2 breast cancer families will provide a high-throughput screening method for identifying families carrying dominant negative mutations in *ATM*
b) Microarray analysis of LCLs from heterozygotes with dominant negative *ATM* mutations, and of stable transfectants generated with mutant *ATM* constructs, will identify a unique set of target genes dysregulated as a consequence of these *ATM* mutations. The information gained can be used to develop an alternate method for high-throughput screening and may also provide candidates for the genes involved in the genesis of ATM-related breast tumours.

This project will establish whether impaired activation of ATM kinase and/or microarray analysis can be used as powerful tool (s) to identify families carrying dominant negative mutations in *ATM*. This will have important clinical ramifications for the families involved in terms of pre-symptomatic diagnosis, surveillance and risk management.

Body

Task 1 - Assay for ATM kinase activation in LCLs from the youngest affected person from 260 high-risk breast cancer families without a pathogenic mutation in BRCA1 and BRCA2 (months 1-6).

At our request, kConFab have established EBV-transformed cell lines from index cases (the youngest affected individual from each family) from 252 high-risk breast cancer families without a pathogenic mutation in BRCA1 or BRCA2. ATM expression was determined by western blotting with anti-ATM antibody and the activation of ATM was measured in vivo using anti-phospho-specific antibodies against the ATM phosphorylation site in p53 (phosphoSer15). Previous work from the laboratory and others have shown that ATM is required only for the immediate and rapid phosphorylation of its targets after ionizing radiation since they are still phosphorylated in ATM-null cells, albeit with delayed kinetics (Khanna et al, 1998, Gatei et al, 2001). Therefore, the activation of ATM was assayed within 30 mins of exposure to ionizing radiation (6 Gy). 42 out of the 252 cell lines tested showed reduced phosphorylation of p53 on Ser15, although the level of expression of ATM was quite comparable to controls. These 42 lines were retested again for Ser15-p53 phosphorylation and only 12 of these showed consistently reduced p53 phosphorylation. We have noticed that p53 phosphorylation does not allow the assessment of ATM heterozygosity with 100% accuracy as it is influenced by cell culture condition and cell density etc. There is a substantial natural variation amongst LCls in both *in vitro* and *in vivo* measures of ATM kinase activity and these assays fail to accurately predict ATM heterozygosity.

Task 2 - Microarray expression profiling with LCLs from 2 families each with members with the T7271G mutation, with and without prior exposure to ionising radiation

Microarray analysis of the T7271G mutation carriers

We carried out extensive microarray experiments on 2 families with the T7271G mutation, Family 1 (Stankovic et al., 1998) and Family 2 (kConFab). RNA was extracted from LCLs from both families containing members with the T7271G mutation with and without prior exposure to ionising radiation. The RNA was pooled into 6 pools on the basis of phenotype:

1) unrelated control individuals with wild type ATM
2) heterozygous carriers of the T7271G mutation from Family 1
3) homozygous for the T7271G mutation from Family 1
4) control individuals with wild type ATM - Family 2 (kConFab)
5) heterozygous carriers of the T7271G mutation who are not affected with breast cancer - Family 2 (kConFab)
6) heterozygous carriers of the T7271G mutation who are affected with breast cancer - Family 2 (kConFab)

Differences in gene expression between these pools were assessed using a total of 46 Compugen 19,000 gene long-oligo microarrays. Each hybridisation was carried out in

triplicate with a dye swap. To understand gene changes that may be present between the ATM pools and to determine whether microarray analysis can be used to identify families carrying mutations in ATM the experiment was divided into 4 sections (outlined in Figure 1):

1. Comparison of different ATM genotypes - heterozygous, homozygous and wild type
2. Comparison of profiles for heterozygotes with disease Vs heterozygotes without cancer, relative to wild type
3. Comparison of all pools via a common wild type ATM reference
4. Pair-wise comparisons of each pool before and after exposure to IR

Data extraction and image analysis of all microarrays was carried out using ImaGene5.0 (BioDiscovery Inc), and data warehousing tools established in house have been used to manage and store data (BASE: Saal *et al.*, 2002; http://microarray.imb.uq.edu.au/BASE). Quality assessment of the microarrays was carried out using "arrayplots" tool in BASE and arrays that failed quality checking were repeated. Subsequent analysis and data mining were performed with BASE and GeneSpring5.5 (Silicon Genetics Inc). The signal of each gene was calculated by the mean signal intensity of each channel with background correction. Normalisation was carried out by print tip Loess, whereby a Loess curve fitted to the Log-intensity versus Log ratio plot was used to adjust the control value for each measurement, if the control channel was lower than 10 then 10 was used instead.

In the case of all direct comparisons, differential expression was defined using a robust statistical methods rather than simple fold change. ANOVA and principle components analysis (PCA) were used to analyse changes in expression between the sample pools, in some cases to reduce discovery of false differential gene expression by ANOVA a Bonferroni multiple testing correction was applied. Genes were also ranked using the B-statistic (Sandiot and Speed's 2001) method where both fold change and variance of signals in replicates is used to determine the likelihood that genes are truly differentially expressed. DAVID (Database for Annotation, Visualization and Integrated Discovery; http://apps1.niaid.nih.gov/david/) and EASEonline (Hosack et al. 2003) were used for biological interpretation of gene lists.

A. Comparison of different ATM genotypes - heterozygous, homozygous and wild type

Family 1 was used to examine differential gene expression between ATM genotypes (heterozygous and homozygous) relative to an unrelated wild type pool (Fig. 1A). Using B-statistics we found 1560 and 774 genes that were differentially expressed in the heterozygotes and homozygotes from Family 1 compared to wild type, representing 8% and 4% of genes on the arrays respectively. A comparison of these differentially expressed genes showed 23 genes that were down regulated in both heterozygotes and homozygotes compared to wild type. These genes were linked to Gene Ontology functional annotations and overrepresentation of protein kinase cascade and NF-kappaB cascade genes was seen with p-values of 0.000174 and 0.000386 respectively. A further 206 genes were overexpressed in both heterozygote and homozygote pools in comparison to the unrelated wild type pool. There were also gene changes that differed between the

heterozygous and homozygous genotype within Family 1, as in a direct comparison of these pools a total of 351 genes were significantly differentially expressed.

B. Comparison of profiles for heterozygotes with breast cancer vs those with no disease, relative to wild type

Carriers of the ATM T7271G mutation in Family 2 were used to analyse changes in gene expression that may be associated with the genesis of ATM-related breast tumours (Fig. 1B). In a direct comparison using B-statistics of heterozygotes with breast cancer and those without, 100 genes differed in their expression. 65 of these were down regulated in the heterozygotes with breast cancer and were linked to Gene Ontology similarity over representation of genes involved in developmental processes with a p-value of 0.0296.
The 2 separate pools of ATM T7271G heterozygotes, those with cancer and those without cancer, were also compared relative to an internal family ATM wild type pool. 35 genes were found to vary significantly using ANOVA with Benjamini and Hochberg with a false discovery rate of 5 % (p-value = 0.05). There was no significance between these genes and Gene Ontology over representation. These data suggest that using LCLs it is difficult to detect any gene changes that may be associated with the development of cancer.

C. Comparison of all family pools via a common wild type reference

The heterozygous, homozygous and wild type pools from both ATM T7271G families were compared to an unrelated wild type control pool for differences in gene expression (Fig. 1C). B-statistics showed that a total of 2068 genes were differentially expressed in at least one pool compared to the unrelated ATM wild type pool. Analysis was carried out to see if these differentially expressed genes are related to ATM genotype. Data was filtered on confidence across replicates using a t-test with a cut off p-value of 0.05 in at least 2 of the 5 conditions. 1,199 genes passed this filter and were used in subsequent analysis. To determine whether any of these genes are related to the ATM T7271G mutation the data was grouped by ATM genotype and ANOVA with Bonferroni correction ($p = 0.005$) was performed. 60 genes showed statistically significant differences in expression between the wild type, heterozygotes and homozygotes pools versus the unrelated ATM wildtype pool. Based on these genes a gene tree using standard correlation was generated (Fig. 2). Clustering of the data resulted in good separation of the wildtype and ATM T7271G cells. Discrete patterns of gene expression were elucidated, including a large group of genes that are over expressed in ATM mutation cells compared to wild type and some that are differentially expressed in ATM T7271G homozygotes exclusively.

Principle component analysis by experiment with the 60 genes above was able to clearly separate wildtype samples from the ATM T7271G carriers with the exception of one comparison (Fig. 3). Two replicates of the pooled RNA from Family 2 with breast cancer were more closely associated with the wildtype family members, however this comparison showed the greatest variation between replicates.

The list of 60 genes that were differentially expressed between the ATM wildtype and T7271G mutation carriers when compared to the entire array gene content show an overrepresentation of genes involved in the response to stress (EASE score of 0.02). Among the 60 genes were 21 genes of unclassified gene ontology, 3 genes that have previously been linked with A-T heterozygotes and others whose association with A-T is novel. The genes that have been associated with ATM heterozygotes include LMO4 and LHX2 are 2 LIM domain genes that had a higher expression in the ATM T7271G cells compared to the wildtype cells. LIM protein over expression in A-T heterozygotes has previously been noted compared to wildtype (Watts et al., 2002). SULT1C2 is also preferentially expressed in the ATM T7271G cells and a close family member SULT1C1 was also over expressed in A-T carriers (Watts et al., 2002).

The majority of genes that differed between the ATM T7271G cells and wildtype cells have not been associated with the ATM gene before and include genes involved in cell growth and/or maintenance, signal transduction and nucleic acid metabolism. A study measuring expression variation between LCLs from normal individuals found that non-variable genes are most heavily represented in signal transduction and cell death/proliferation (Cheung et al. 2003), which suggests that these genes are linked to A-T and not due to individual variation. Amongst the genes involved in cell growth and/or maintenance are RanBP1 (Ran-binding protein 1), CCNB1 (cyclin B1) and BCCIP (BRCA2 and CDKN1A (p21) interacting protein), which are all expressed at higher levels in the T7271G cells compared to the wildtype cells. RanBP1 is a major effector of Ran, which is involved in spindle formation during mitosis. RanBP1 over expression yields abnormal mitotic spindles with multiple poles (Di Fiore et al. 2003), potentially causing loss of genomic integrity, which is a common feature in cancer. Another gene BCAS4 (Breast carcinoma amplified sequence), was over expressed in the ATM T7271G heterozygotes and homozygote cell lines compared to the wildtype pool but showed no differential expression between wildtype cells. This gene is located in a chromosomal region that is frequently amplified in breast caner, 20q13 and BCAS4 itself is known to be amplified in a large number of breast carcinoma cell lines (Barlund et al. 2002). Thus over expression of the BCAS4 gene in cells that have a mutation in the ATM gene which predisposes women to breast cancer is interesting.

D. Pairwise comparison of each pool before and after exposure to IR

Carriers of the T7271G ATM mutation have been shown to have an increased sensitivity to ionising radiation (Stankovic et al., 1998). To assess how the T7271G ATM mutation may affect how cells respond to ionising radiation, we we irradiated LCLs from both families and harvested RNA from unirradiated and from cells treated with 3 Gy IR. RNA was pooled from irradiated and unirradiated cells within each family on the basis of ATM T7271G mutation status. Pooled unirradiated and irradiated samples were labelled and hybridised together onto the 19K human chips (Fig. 1D).

There was a difference in the response of ATM wildtype and T7271G mutation carriers 12 hours after 3 Gy IR. The ATM T7271G cells showed fewer significant gene changes in response to IR, this has not previously been reported and may be a contributing factor

in the increased sensitivity of these cells to IR. B-statistics was performed to identify genes that were changing 12 hrs after IR treatment in each RNA pool. The fewest significant changes were found in the T7271G homozygote pool from Family 1, whilst the experiments with the highest number of gene changes after IR were the ATM wild type RNA pools. When the gene changes were grouped on ATM T7271G mutation status a group of 63 genes were identified that were differentially expressed specifically in both the Family 2 ATM wild type pool and the unrelated wild type pool after IR but not in the ATM T7271G RNA pools. All of the 63 genes were down regulated 12 hr after 3 Gy IR. These ATM-dependant down regulated genes were linked to Gene Ontology and an over representation of genes involved in DNA replication and cell cycle regulation was found (p-values of 0.000459 and 0.0152 respectively). Twenty one of these genes were also detected by ANOVA with Benjamini and Hochberg False Discovery Rate (p-value cutoff 0.01), which detected 99 genes that were differentially expressed between the wild type and A-T cells.

The 63 genes was identified that were differentially expressed specifically in the ATM wild type cells after IR but not in the RNA pooled from ATM T7271G cells, including some genes of unknown function while others have not previously been associated with ATM or DNA damage repair. Many of the discriminatory genes are associated with DNA damage repair and cell cycle and were down regulated in the wild type cells 12 hr after IR. This is consistent with Heinloth et al. (2003) who found the majority of genes affected by exposure to IR were down regulated 6 hr after treatment, were involved with DNA repair and were expressed in an ATM-dependant manner.

Independent validation of the array data by real time PCR was performed on 7 of the genes: RFC5, POLD1, MCM7, CDK8, CCND2, CDC2 and ANAPC5. Validation was performed on RNA from the pooled samples, individual members of the pools and RNA from LCLs that were not used in the array experiments. All real time PCR reactions were normalized to an internal control gene (HPRT), and then to self that was not treated with IR. The pooled RNA samples all validated and there was good correlation between the array data and real time PCR data (Fig. 4). When individuals from the pools were analysed by real time PCR 6/7 genes were confirmed and showed good correlation with array results, POLD1 did not validate as its expression did not change in the wild type individual after IR. Expression levels of these 7 genes after IR was also analysed by real time PCR in LCLs heterozygous for the T7271G mutation that were not used in the micrarrays and also LCLs that are heterozygous for different ATM mutations causing a truncation of the ATM gene. The heterozygous carriers of T7271G mutation display a gene expression phenotype that appears identical to carriers of protein truncating mutations in *ATM,* suggesting that the expression signature may not be specific for this particular mutation in ATM but rather can be used to identify ATM heterozygosity in general.

Transcript profiling is a good way of determining molecular alterations. However for functional relevance, protein levels are important as RNA may be subject to splicing or post transcriptional modification. Therefore to confirm that transcript level correlates with protein abundance few of the genes used for validation by real time PCR were

selected for validation by Western blotting. The protein levels of these were shown to go down 12 hours after 3 Gy IR in the ATM wild type cells but not the ATM T7271G mutation cells, in agreement with the RNA from the array and real time PCR data. A manuscript describing the results obtained under Task 2 is being prepared for submission.

Task 3 – Engineer mutant constructs and generate transfections with wild-type and mutant constructs.

We have constructed the pathogenic *ATM* missense mutation (V2424G; T7271G), which confers a high risk of breast cancer, using cDNA mutagenesis. The mutant and wild-type ATM cDNAs were also tagged with a Green-Fluorescent Protein at their N-terminus. ATM-null cells were stably transfected with ATM cDNA constructs and ATM expression and kinase activity in terms of ability to phosphorylate various targets of ATM *in vitro* and *in vivo* was determined. The overall summary of results was that the mutant form of ATM was unable to phosphorylate most of the wild-type ATM targets. A recent study has linked the activation of ATM to its autophosphorylation (Bakkenist and Kastan, 2003). It has been shown that ATM exists as an inactive dimer in cells and exposure to irradiation causes auto phosphorylation on Serine 1981 leading to dimer dissociation and its activation as a kinase. The subsequent monomers then result in rapid and immediate phosphorylation of targets. Therefore, we compared the ability of the wild-type and the mutant ATM protein to auto-phosphorylate. Data presented in the Fig 5A shows that both the wild-type and the mutant ATM have comparable auto phosphorylation activity as assessed by *in vivo* phosphorylation on Ser1981 after IR, however the mutant form of ATM is defective in phosphorylation of p53, H2AX and multiple other targets of ATM analysed western blotting and immunofluorescence (Fig 5A and B). In addition, Ser1981A mutant form of ATM, which is defective in auto-phosphorylation, is fully competent to phosphorylate ATM targets *in vivo*. Taken together the data suggests that failure to observe phosphorylation of substrates is not caused by lack of auto-phosphorylation of mutant ATM protein. This mutant form of ATM appears to separate the auto-phosphorylation and substrate phosphorylation functions of ATM. We are currently investigating the mechanistic basis of this separation of function effect before the submission of this work for publication.

Task 4 – Extend the analysis of ATM kinase activity to all available family members based on results with the index cases.

LCLs from 7271 and IVS106T-G families have been established and all the family members were genotyped for mutations and LCLs of all mutation carriers and some of the non-carriers (normals) in the family were tested for ATM activity in *in-vitro* and *in-vivo*. The data obtained was inconclusive as 1 of the 9 cell lines tested from non-carriers had reduced activity whereas half of the carriers tested had normal ATM activity.

Task 5 – Start mutation analysis of the ATM gene in families with compromised ATM kinase activity.

Because of the variability of results with ATM kinase activity mentioned under task 1, it was not possible to identify accurately the cell lines with reduced ATM activity.

Therefore, to identify additional families that might carry mutation in ATM, we have screened index cases from 80 families for mutations in ATM by sequencing and have identified one extra T7271G family and have shown that the variant segregates with the disease in this family. We will validate the expression profile in an LCL from this additional 7271T>G family by RT-PCR. We have also found two rare variants of ATM (7187T>G and 8158G>C) which were absent in 450 controls. It is therefore possible that these very rare variants are breast cancer related. We are therefore seeking additional biospecimen (germline DNA and tumour blocks) from the two carrier families to determine whether the variants segregate with disease, and undergo loss of heterozygosity. In addition, we will profile LCLs from these carriers to determine whether they show the characteristic expression profile of ATM mutation carriers.

Key Research Accomplishments

1 252 LCLs have been established from index cases from non-BRCA1/2 breast cancer families.
2 All LCLs have been analysed for ATM expression and kinase activity using anti-phospho Ser15 antibody.
3 12/252 (4.7%) LCLs showed markedly reduced ATM kinase activity, despite normal level of expression of ATM protein.
4 Microarray profiling of two ATM T7271G families containing heterozygous, homozygous and wild type members
5 Data extraction, image analysis and data management of microarrays
6 Analysis of gene changes occurring between different ATM genotypes
7 Comparison of profiles for T7271G ATM heterozygotes with or without breast cancer
8 Analysis of whether ATM genotype may affect how cells respond to ionising radiation
9 Validation of the microarrays using real-time PCR
10 Identification of one additional T7271G family
11 Identification of two rare variants of ATM that might be breast cancer related

Reportable outcomes

A: Publications

1. Khanna, K.K. and Georgia Chenevix-Trench. ATM and Genome Maintenance: defining its role in breast cancer susceptibility. *The Journal of Mammary Gland Biology and Neoplasia*, 2004 Jul;9(3):247-62.
2. Waddell, N et al. Identification of ATM mutation carriers using expression array analysis. In Preparation.
3. Young et al. Functional analysis of breast cancer associated mutation in ATM. In preparation.

B: Conference presentation

- Presented at the 3rd Australian Microarray Conference, Couran Cove, Australia July 23rd-26th 2003
- Presented at the KConFab and Australian Ovarian Cancer Study (AOCS) and Family Cancer Clinics of Australia and New Zealand, Couran Cove, Australia Aug 18th-21st 2004

Conclusions

252 lymphoblastoid cell lines from index cases of multiple-case breast cancer families were screened for ATM kinase activity and 12 were identified with markedly reduced ATM kinase activity. However, there is a substantial natural variation amongst LCls in both *in vitro* and *in vivo* measures of ATM kinase activity and these assays fail to accurately predict ATM heterozygosity. Moreover, when carriers and non-carrier of mutation from T7271G family were tested for ATM activity, half of the carriers had normal activity while some of the non-carriers (normals) in the family had reduced activity. Overall, analysis of the data suggests that impaired activation of ATM can not be used as a screening tool to identify families that carry dominant negative mutation in ATM due to natural variation amongst LCLs.

Microarrays have been performed to determine differences in gene expression between heterozygotes (with or without breast cancer) or homozygotes for the T7271G mutation against each other and wild type control pools. The response of these different cells to ionising radiation has also been investigated. Although, there is substantial natural variation both in gene expression among individuals and in their transcriptional response to IR, however, these differences are thought to be genetic as the variance decrease within families (Cheung et al. 2003). This makes the ATM T7271G families used in this study a useful tool for measuring gene changes associated with the ATM mutation and the response to IR, since variation between family members will be smaller and gene changes detected in both A-T families are more likely to be linked to the mutation. The data from the 2 families has suggested candidate genes that have altered expression in T7271G *ATM* mutation carriers, and how these mutations affect the cells response to IR. The validation of this data in carriers of different ATM mutation indicated that the heterozygous carriers of T7271G mutation display a gene expression phenotype that appears identical to carriers of protein truncating mutations in *ATM,* suggesting that the expression signature may not be specific for this particular mutation in ATM but rather can be used to assess the downstream effects of any germ-line change in the ATM gene. However from the data obtained with LCLs, it is difficult to determine what genes may be involved in disease state and the genesis of ATM-related breast tumours.

References

Broeks A, Urbanus JH, Floore AN, et al: ATM-heterozygous germline mutations contribute to breast cancer- susceptibility. Am J Hum Genet 66:494-500., 2000

Barlund M, Monni O, Weaver JD, Kauraniemi P, Sauter G, Heiskanen M, Kallioniemi OP, Kallioniemi A. Cloning of BCAS3 (17q23) and BCAS4 (20q13) genes that undergo

amplification, overexpression, and fusion in breast cancer. Genes Chromosomes Cancer. 35(4):311-7 (2002)

Chenevix-Trench G, Spurdle AB, Gatei M, et al. Dominant negative ATM mutations in breast cancer families. J Natl Cancer Inst. 94:205-15., 2002.

Cheung VG, Conlin LK, Weber TM., Arcaro M, Jen K-Y, Morley M and Spielman RS. Natural variation in human gene expression assessed in lymphoblastoid cells. Nature Genetics 33: 422-425 (2003)

Di Fiore B, Ciciarello M, Mangiacasale R, Palena A, Tassin AM, Cundari E, Lavia P. Mammalian RanBP1 regulates centrosome cohesion during mitosis. Journal of Cell Science 1;116:3659 (2003)

Gatei, M, Zhou, BB, Karen H, et al: ataxia telangiectasia mutated (ATM) kinase and ATM and Rad3 related kinase mediate phosphorylation of Brca1 at distinct and overlapping sites. In vivo assessment using phospho-specific antibodies. J Biol Chem. 276:17276-80, 2001.

Heinloth AN, Shackelford RE, Innes CL, Bennett L, Li L, Amin RP, Sieber SO, Flores KG, Bushel PR, Paules RS. ATM-dependent and -independent gene expression changes in response to oxidative stress, gamma irradiation, and UV irradiation. Radiation Research 160(3): 273-90 (2003)

Jen K and Cheung V: Transcriptional response to lymphoblastoid cells to ionizing radiation. Genome Research 2092-2100 (2003)

Khanna KK, Keating KE, Kozlov S, et al: ATM associates with and phosphorylates p53: mapping the region of interaction. Nat Genet 20:398-400, 1998

Saal LH, Troein C, Vallon-Christersson J, Gruvberger S, Borg A and Peterson C: BioArray Software Environment: A Platform for Comprehensive Management and Analysis of Microarray Data. Genome Biology 3:8 (2002)

Stankovic T, Kidd AM, Sutcliffe A, et al: ATM mutations and phenotypes in ataxia-telangiectasia families in the British Isles: expression of mutant ATM and the risk of leukemia, lymphoma, and breast cancer. Am J Hum Genet 62:334-45 (1998)

Watts JA, Morley M, Burdick JT, Fiori JL, Ewens WJ, Spielman RS and Cheung VG. Gene Expression Phenotype in Heterozygous Carriers of Ataxia Telangiectasia. American Journal of Human Genetics 71:791-800 (2002).

Figure legends

Figure 1. Microarray experimental design to analyse changes between the pooled ATM members (heterozygous m/+, homozygous m/m and wildtype +/+). Arrows between the pooled samples represent a single microarray, the point of each arrow indicates the sample labelled with Cy3, microarrays were carried out triplicate with one dye swap.
A. A comparison of ATM heterozygous and homozygous from Family 1 via an unrelated ATM wild type pool
B. To detect genes that may be involved in the genesis of ATM-related breast tumours a comparison of profiles for heterozygotes with cancer Vs those with no breast cancer relative to wild type was performed
C. An unrelated wild type control pool was used as a common reference to compare all pools
D. To assess how ATM mutations may affect the cells response to ionising radiation a pair-wise comparison of each pool before and after exposure to ionising radiation was performed

Figure 2. A gene tree of 60 genes with statistically significant differences between wild type ATM pools (+/+), heterozygote (+/-) and homozygote (-/-) T7271G ATM pools from the 2 families. ANOVA with Bonferoni correction ($p = 0.005$) was carried out on a list of filtered genes. The 60 genes that were found to have statistically significant differences between ATM genotype were clustered based on expression data.

Figure 3 Principle components analysis of the 60 genes with statistically significant differences between wild type ATM pools, heterozygote and homozygote T7271G ATM pools from the 2 families.

Figure 4. Validation of the microarray data by real time PCR. 77 genes were down regulated after 3 Gy IR in the wildtype RNA pools, 7 of these were selected for real time PCR. The microarray data and real time PCR are shown for each gene.

Figure 5: Immunoblot showing expression, autophosphorylation of ATM (pS1981) and p53 phosphorylation in AT1ABR cells (an A-T lymphoblastoid cell line) stably expressing GFP-ATM wild-type (wt), kinase-dead (KD), V2424G mutant. Whole cell extracts were prepared from cells 30 mins after exposure to 6 Gy IR. B) Fluorescent microscopy with anti-H2AX Ser139 antibody with exposure to IR (30 mins after 4Gy). Cells were fixed with paraformaldehyde then permeablised with 0.2% Triton X-100 prior to staining. DNA was visualized by staining with Dapi.

Fig. 1 Experimental Design

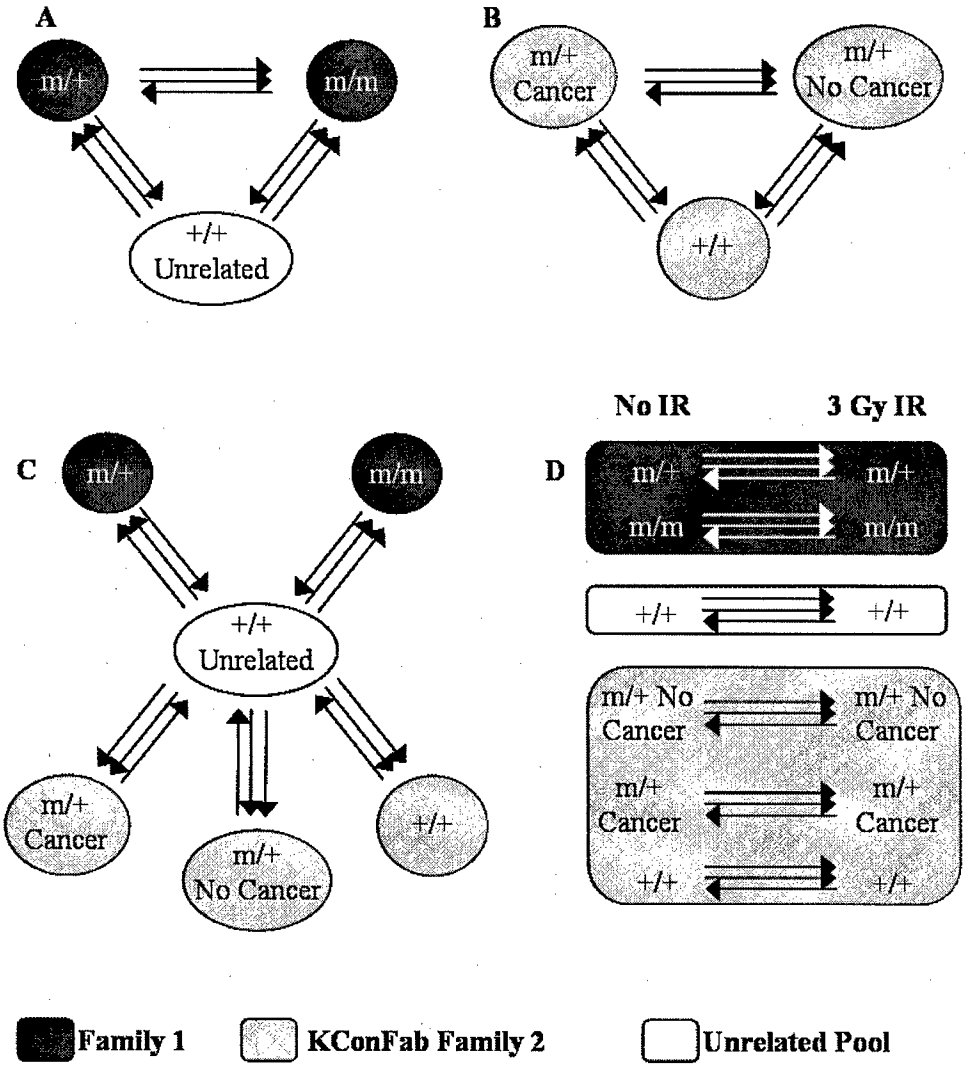


Fig. 2 **Hierarchical Clustering of Genes Associated with ATM Genetic Characteristic**

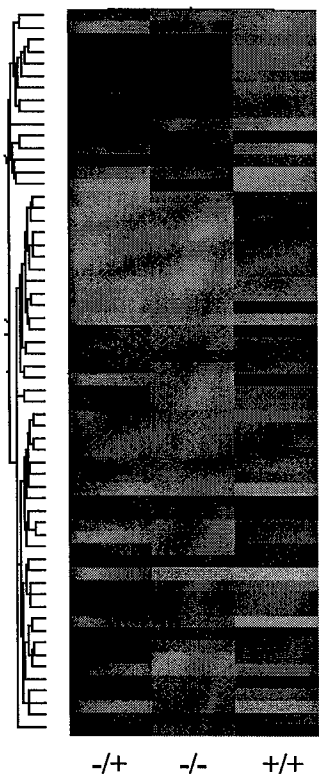
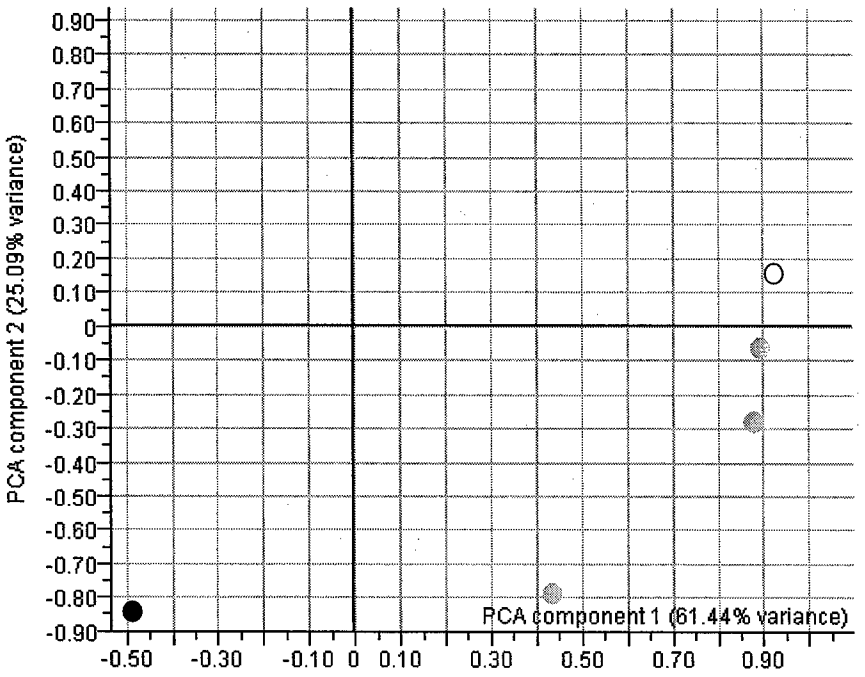


Figure 3. Principle Components Analysis of Genes Associated with ATM Genetic Characteristic



● ATM wildtype

● ATM T7271G heterozygote

○ ATM T7271G homozygote

Figure 4. Validation of Microarray data with realtime PCR

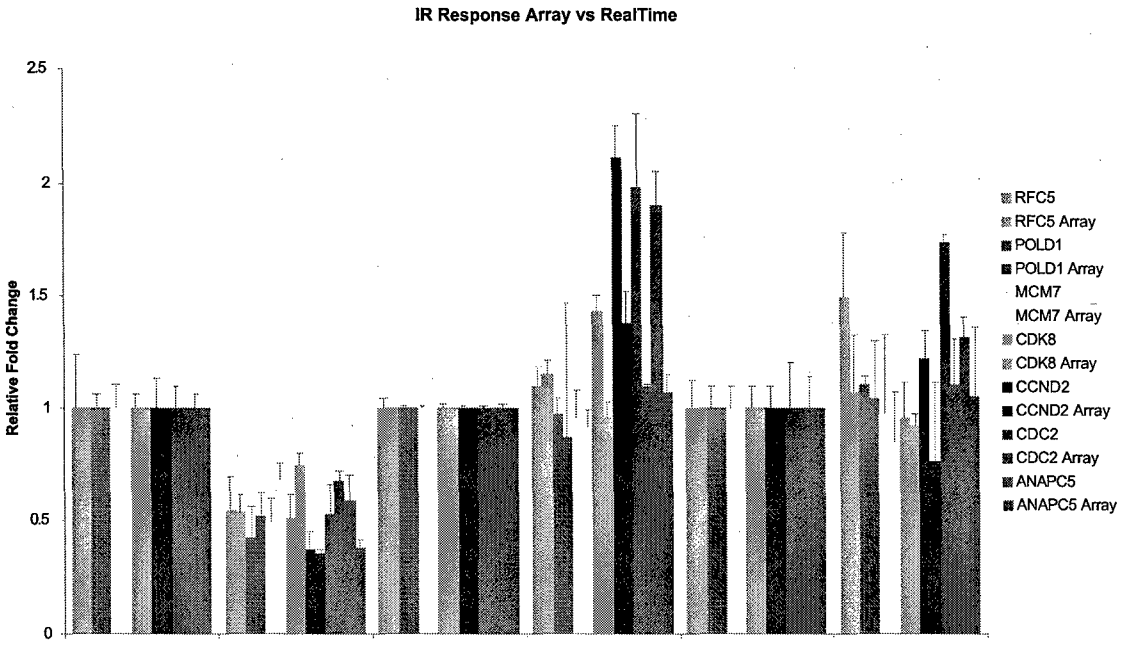


Fig. 5A

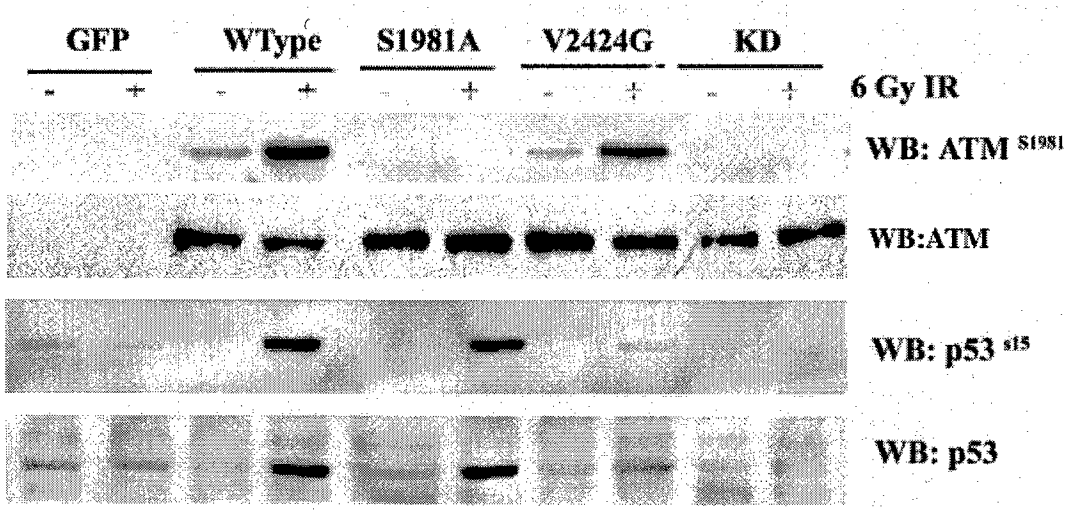
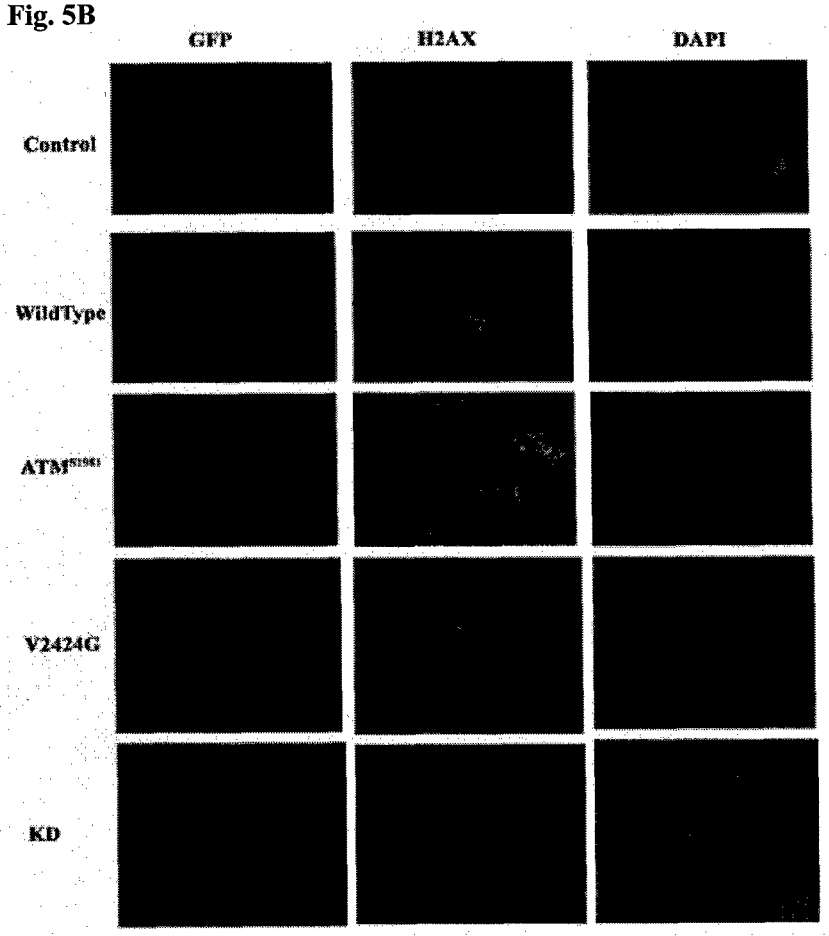

GFP
WType
S1981A
V2424G
KD
- + - + - + - + - +
6 Gy IR
WB: ATM S1981
WB:ATM
WB: p53 S15
WB: p53

Fig. 5B



V2424G mutant cannot phosphorylate substrate